

Isolated DNA sequence capable of serving as regulatory element in a chimeric gene which can be used for the transformation of plants.

The present invention relates to the use of a
5 regulatory element isolated from transcribed plant
genes, of new chimeric genes containing them and to
their use for the transformation of plants.

Numerous phenotypic characters associated with the expression of one or more gene elements can be integrated into the genome of plants and thus confer on these transgenic plants advantageous agronomic properties. In a nonexhaustive manner, there may be mentioned: the resistances to pathogenic agents for crops, the resistance to phytotoxic plant-protection products, the production of substances of dietary or pharmacological interest. In addition to the isolation and characterization of the gene elements encoding these various characters, an appropriate expression should be ensured. This appropriate expression may be situated both at the qualitative and quantitative levels. At the qualitative level, for example the spatial level: preferential expression in a specific tissue, or temporal level: inducible expression; at the quantitative level, by the accumulated quantity of the product of expression of the gene introduced. This appropriate expression depends, for a large part, on the presence of regulatory gene elements associated with the transgenes, in particular as regards the

quantitative and qualitative elements. Among the key elements ensuring this appropriate regulation, the use of single or combined homologous or heterologous promoter elements has been widely described in the scientific literature. The use of a regulatory element downstream of the transgene was used for the sole purpose of putting a boundary which makes it possible to stop the process of transcription of the transgene, without presupposition as to their role as regards the quality or the quantity of the expression of the transgene.

The present invention relates to the use of an intron 1 isolated from plant genes as a regulatory element, of new chimeric genes containing them and to their use for the transformation of plants. It relates to an isolated DNA sequence capable of serving as a regulatory element in a chimeric gene which can be used for the transformation of plants and allowing the expression of the product of translation of the chimeric gene in particular in the regions of the plant undergoing rapid growth, which comprises, in the direction of transcription of the chimeric gene, at least one intron such as the first intron (intron 1) of the noncoding 5' region of a plant histone gene. It relates more particularly to the simultaneous use of the intron 1 as a regulatory element and of promoters isolated from the same plant gene. It allows the appropriate expression, both quantitative and

qualitative, of the transg n's under the control of these elements for gene regulation. This appropriate expression, obtained by the use of the present invention, may relate to characters such as: the 5 resistance to pathogenic agents for crops, the resistance to phytotoxic plant-protection products, the production of substances of dietary or pharmacological interest. In particular, it makes it possible to confer on the transgenic plants an enhanced tolerance to 10 herbicides by a qualitative and quantitative preferential expression of the product of expression of the chimeric genes in the regions of the plant undergoing rapid growth. This specific appropriate expression of the gene for herbicide resistance is 15 obtained by the simultaneous use of the promoter regulatory elements and of at least one intron 1 of the histone gene of the "H3.3- like" type as regulatory element. Such a pattern of expression can be obtained for all the characters which are of interest, as 20 described above, with the regulatory elements used to confer an enhanced herbicide tolerance. The present invention also relates to the plant cells transformed with the aid of these genes and the transformed plants regenerated from these cells as well as the plants 25 derived from crossings using these transformed plants.

Among the plant-protection products used for the protection of crops, the systemic products are characterized in that they are transported in the plant

after application and, for some of them, accumulate in the parts undergoing rapid growth, especially the caulinary and root apices, causing, in the case of herbicides, deterioration, up to the destruction, of the sensitive plants. For some of the herbicides exhibiting this type of behaviour, the primary mode of action is known and results from inactivation of characterized enzymes involved in the biosynthesis pathways of compounds required for proper development of the target plants. The target enzymes of these products may be located in various subcellular compartments and observation of the mode of action of known products most often shows a location in the plastid compartment.

Tolerance of plants sensitive to a product belonging to this group of herbicides, and whose primary target is known, may be obtained by stable introduction, into their genome, of a gene encoding the target enzyme, of any phylogenetic origin, mutated or otherwise with respect to the characteristics of inhibition, by the herbicide, of the product of expression of this gene. Another approach comprises introducing, in a stable manner, into the genome of sensitive plants a gene of any phylogenetic origin encoding an enzyme capable of metabolizing the herbicide into a compound which is inactive and nontoxic for the development of the plant. In the

latter case, it is not necessary to have characterized the target of the herbicide.

Given the mode of distribution and accumulation of products of this type in the treated plants, it is advantageous to be able to express the product of translation of these genes so as to allow their preferential expression and their accumulation in the regions of the plant undergoing rapid growth where these products accumulate. Furthermore, and in the case where the target of these products is located in a cellular compartment other than the cytoplasm, it is advantageous to be able to express the product of translation of these genes in the form of a precursor containing a polypeptide sequence allowing directing of the protein conferring the tolerance into the appropriate compartment, and in particular in the plastid compartment.

By way of example illustrating this approach, there may be mentioned glyphosate, sulfosate or 20 fosametine which are broad-spectrum systemic herbicides of the phosphonomethylglycine family. They act essentially as competitive inhibitors, in relation to PEP (phosphoenolpyruvate), of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19). After their 25 application to the plant, they are transported into the plant where they accumulate in the parts undergoing rapid growth, especially the caulinary and root apices.

causing the deterioration, up to the destruction, of the sensitive plants.

GPAPS, the principal target of these products, is an enzyme of the pathway of biosynthesis of aromatic amino acids which is located in the plastid compartment. This enzyme is encoded by one or more nuclear genes and is synthesized in the form of a cytoplasmic precursor and then imported into the plastids where it accumulates in its mature form.

10 The tolerance of plants to glyphosate and to
products of the family is obtained by the stable
introduction, into their genome, of an EPSPS gene of
plant or bacterial origin, mutated or otherwise with
respect to the characteristics of inhibition, by
15 glyphosate, of the product of this gene. Given the mode
of action of glyphosate, it is advantageous to be able
to express the product of translation of this gene so
as to allow its high accumulation in the plastids and,
furthermore, in the regions of the plant undergoing
20 rapid growth where the products accumulate.

It is known, for example, from American patent 4,535,060 to confer on a plant a tolerance to a herbicide of the above type, in particular N-phosphonomethylglycine or glyphosate, by introduction, into the genome of the plants, of a gene encoding an EPSPS carrying at least one mutation making this enzyme more resistant to its competitive inhibitor (glyphosate), after location of the enzyme in the

plastid compartment. These techniques require, however, to be improved for greater reliability in the use of these plants during a treatment with these products under agronomic conditions.

5 In the present description, "plant" is understood to mean any differentiated multicellular organism capable of photosynthesis and "plant cell" any cell derived from a plant and capable of constituting undifferentiated tissues such as calli, or

10 differentiated tissues such as embryos or plant portions or plants or seeds. "Intron 1 of *Arabidopsis* as a regulatory element" is understood to mean an isolated DNA sequence of variable length, situated upstream of the coding part or corresponding to the

15 structural part of a transcribed gene. Gene for tolerance to a herbicide is understood to mean any gene, of any phylogenetic origin, encoding either the target enzyme for the herbicide, optionally having one or more mutations with respect to the characteristics

20 of inhibition by the herbicide, or an enzyme capable of metabolizing the herbicide into a compound which is inactive and nontoxic for the plant. Zones of the plants undergoing rapid growth are understood to mean the regions which are the seat of substantial cell

25 multiplications, in particular the apical regions.

The present invention relates to the production of transformed plants having an enhanced tolerance to herbicides accumulating in the zones of

th treated plants undergoing rapid growth, by regeneration of cells transformed with the aid of new chimeric genes comprising a gene for tolerance to these products. The subject of the invention is also the production of transformed plants having an enhanced tolerance to herbicides of the phosphonomethylglycine family by regeneration of cells transformed with the aid of new chimeric genes comprising a gene for tolerance to these herbicides. The invention also relates to these new chimeric genes, as well as to transformed plants which are more tolerant because of a better tolerance in the parts of these plants undergoing rapid growth, as well as to the plants derived from crossings using these transformed plants. Its subject is also new intron 1 of a plant histone and its use as regulatory zone for the construction of the above chimeric genes.

More particularly, the subject of the invention is a chimeric gene for conferring on plants especially an enhanced tolerance to a herbicide having EPSPS as target, comprising, in the direction of transcription, a promoter element, a signal peptide sequence, a sequence encoding an enzyme for tolerance to the products of the phosphonomethylglycine family and a regulatory element, characterized in that the regulatory element comprises a fragment of an intron 1 of a plant histone gene in any orientation relative to its initial orientation in the gene from which it is

derivative, all wing the preferential expression and the accumulation of the protein tolerance to the herbicide in the zones for accumulation of the said herbicide.

5 The histone gene, from which intron 1
according to the invention is derived, comes from a
monocotyledonous plant such as for example wheat, maize
or rice, or preferably from a dicotyledonous plant such
as for example lucerne, sunflower, soya bean, rapeseed
10 or preferably Arabidopsis thaliana. Preferably, a
histone gene of the "H3.3-like" type is used.

The signal peptide sequence comprises, in the direction of transcription, at least one signal peptide sequence of a plant gene encoding a signal peptide directing transport of a polypeptide to a plastid, a portion of the sequence of the mature N-terminal part of a plant gene produced when the first signal peptide is cleaved by proteolytic enzymes, and then a second signal peptide of a plant gene encoding a signal peptide directing transport of the polypeptide to a sub-compartment of the plastid. The signal peptide sequence is preferably derived from a gene for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) according to European patent application PCT 508 909. The role of this characteristic sequence is to allow the release, into the plastid compartment, of a mature polypeptide with maximum efficiency, preferably in a native form.

The coding sequence which can be used in the chimeric gene according to the invention comes from a herbicide tolerance gene of any phylogenetic origin. This sequence may be especially that of the mutated 5 EPSPS having a degree of tolerance to glyphosate.

The promoter element according to European patent application PCT 507 698 may be of any origin, in a single or duplicated or combined form of a gene naturally expressed in plants, that is to say, for example of bacterial origin such as that of the nopaline synthase gene, or of viral origin such as that of the 35S transcript of the cauliflower mosaic virus, or preferably of plant origin such as that of the small subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase or preferably such as that of a plant histone gene and preferably from Arabidopsis thaliana. A histone gene of the "H4" type is preferably used.

The chimeric gene according to the invention
20 may comprise, in addition to the above essential parts,
an untranslated intermediate zone (linker) between the
promoter zone and the coding zone as well as between
the coding zone and intron 1 and which may be of any
phylogenetic origin.

25 The following examples show by way of illustration, but with no limitation being implied, several aspects of the invention: isolation of the introns according to the invention and their use for

th genetic transformation of plants as well as the improved qualities of expression of the heterologous genes of plants transformed with the aid of these introns. References to "Current Protocols in Molecular Biology" are to Volumes 1 and 2, Ausubel F.M. et al., published by Greene Publishing Associates and Wiley Interscience (1989) (CPMB).

EXAMPLE 1:

1. Production of an EPSPS fragment from

10 Arabidopsis thaliana

a) two 20-mer oligonucleotides of respective

sequences:

5'-GCTCTGCTCATGTCCTGCTCC-3'

5'-GCCCGCCCTTGACAAAAGAAA-3'

15 were synthesized from the sequence of an EPSPS gene from Arabidopsis thaliana (Klee H.J. et al., (1987) Mol. Gen. Genet., 210, 437-442). These two oligonucleotides correspond to positions 1523 to 1543 and 1737 to 1717, respectively, of the published sequence and in convergent orientation.

20 b) The total DNA from Arabidopsis thaliana

(var. columbia) was obtained from Clontech (catalogue reference: 6970-1)

25 c) 50 nanograms (ng) of DNA are mixed with 300 ng of each of the oligonucleotides and subjected to 35 amplification cycles with a Perkin-Elmer 9600 apparatus under the standard medium conditions for

amplification command by the supplier. The resulting 204 bp fragment constitutes the GFPSPS fragment from *Arabidopsis thaliana*.

2. Construction of a library of a cDNA from a 5 BMS maize cell line.

a) 5 g of filtered cells are ground in liquid nitrogen and the total nucleic acids extracted according to the method described by Shure et al. with the following modifications:

10 - the pH of the lysis buffer is adjusted to
pH = 9.0;

- after precipitation with isopropanol, the pellet is taken up in water and after dissolution, adjusted to 2.5M LiCl. After incubation for 12 h at $[lacuna]0^{\circ}\text{C}$, the pellet from the 15 min centrifugation at 30,000 g at 4°C is resolubilized. The LiCl precipitation stage is then repeated. The

resolubilized pellet constitutes the fraction of the total nucleic acids.

b) the RNA-poly A+ fraction of the RNA fraction is obtained by chromatography on an oligo-dT cellulose column as described in "Current Protocols in Molecular Biology".

25 c) Synthesis of double-stranded cDNA with an
EcoRI synthetic end: it is carried out by following the
procedure of the supplier of the various reagents

九月九日望蜀王山

necessary for this synthesis is in the form of a kit: the "copy kit" from the company Invitrogen.

Two single-stranded and partially complementary oligonucleotides of respective sequences:

5' -AATTCCCCGGG-3'

5'-CCCGGG-3' (the latter being
phosphorylated)

are ligated to double-stranded cDNAs with blunt ends.

This ligation of the adaptors results in the
10 creation of SmaI sites attached to the double-stranded
cDNAs and of EcoRI sites in cohesive form at each end
of the double-stranded cDNAs.

d) Creation of the library:

The cDNAs having at their ends the cohesive
15 artificial EcoRI sites are ligated to the λgt10
bacteriophage cDNA cut with EcoRI and dephosphorylated
according to the procedure of the supplier New England
Biolabs.

An aliquot from the ligation reaction was
20 encapsidated in vitro with encapsidation extracts:
Gigapack Gold according to the supplier's instructions,
this library was titrated using the bacterium *E.coli*
c600hfl. The library thus obtained is amplified and
stored according to the instructions of the same
25 supplier and constitutes the cDNA library from BMS
maize cell suspension.

3. Screening of the cDNA library from EMS maize cell suspension with the NPSPS probe from Arabidopsis thaliana:

The procedure followed is that of "Current Protocols in Molecular Biology". Briefly, about 10^6 recombinant phages are plated on an LB plate at a mean density of 100 phages/cm². The lysis plaques are replicated in duplicate on a Hybond N membrane from Amersham.

10 The DNA was fixed onto the filters by a 1600
kJ UV treatment (Stratalinker from Stratagene). The
filters were prehybridized in: 6xSSC/0.1 % SDS/0.25
[lacuna] skimmed milk for 2 h at 65°C. The EPSPS probe
15 from Arabidopsis thaliana was labelled with ³²P-dCTP by
random priming according to the instructions of the
supplier (Kit Ready to Go from Pharmacia). The specific
activity obtained is of the order of 10⁸ cpm per µg of
fragment. After denaturation for 5 min at 100°C, the
probe is added to the prehybridization medium and the
20 hybridization is continued for 14 hours at 55°C. The
filters are fluorographed for 48 h at -80°C with a
Kodak XAR5 film and intensifying screens Hyperscreen
RPN from Amersham. The alignment of the positive spots
25 on the filter with the plates from which they are
derived make it possible to collect, from the plate,
the zones corresponding to the phages exhibiting a
positive hybridization response with the EPSPS probe
from Arabidopsis thaliana. This step of plating,

transfer, hybridization and recovery is repeated until all the spots of the plate of phages successively purified prove 100 % positive in hybridization. A lysis plaque per independent phage is then collected in the diluent λ medium (Tris-Cl pH=7.5; 10 mM MgSO₄; 0.1M NaCl; 0.1 % gelatine), these phages in solution constituting the positive EPSPS clones from the BMS maize cell suspension.

4. Preparation and analysis of the DNA of the EPSPS clones from the BMS maize cell suspension.

About 5x10⁸ phages are added to 20 ml of C600hfl bacteria at OD 2 (600 nm/ml) and incubated for 15 minutes at 37°C. This suspension is then diluted in 200 ml of growth medium for the bacteria in a 1 l Erlenmeyer flask and shaken in a rotary shaker at 250 rpm. Lysis is observed by clarification of the medium, corresponding to lysis of the turbid bacteria and occurs after about 4 h of shaking. This supernatant is then treated as described in "Current Protocols in Molecular Biology". The DNA obtained corresponds to the EPSPS clones from the BMS maize cell suspension.

One to two µg of this DNA are cut with EcoRI and separated on a 0.8 % LGTA/TBE agarose gel (ref. CPMB). A final verification consists in ensuring that the purified DNA indeed exhibits a hybridization signal with the EPSPS probe from Arabidopsis thaliana. After electrophoresis, the DNA fragments are

4000223333 - 120001
TOP SECRET

transferred nt Hybond N membran fr m Am rsham
according to the Southern proc dure d scribed in
"Current Protocols in Molecular Biology". The filter is
hybridized with the EPSPS probe from Arabidopsis
5 thaliana according to the conditions described in
paragraph 3 above. The clone exhibiting a hybridization
signal with the EPSPS probe from Arabidopsis thaliana
and containing the longest EcoRI fragment has a gel-
estimated size of about 1.7 kbp.

10 5. Production of the PRPA-ML-711 clone:

Ten μ g of DNA from the phage clone containing
the 1.7 kbp insert are digested with EcoRI and
separated on a 0.8 % LGTA/TBE agarose gel (ref. CPMB).
The gel fragment containing the 1.7 kbp insert is
15 excised from the gel by BET staining and the fragment
is treated with β -agarase according to the procedure of
the supplier New England Biolabs. The DNA purified from
the 1.7 kbp fragment is ligated at 12°C for 14 h with
DNA from the plasmid pUC 19 (New England Biolabs) cut
20 with EcoRI according to the ligation procedure
described in "Current Protocols in Molecular Biology".
Two μ l of the above ligation mixture are used for the
transformation of one aliquot of electrocompetent
E.coli DH10B; the transformation occurs by
25 electroporation using the following conditions: the
mixture of competent bacteria and ligation medium is
introduced into an electroporation cuvette 0.2 cm thick
(Biorad) previously co l d to 0°C. The physical

1000238800 - 1002200

lectroporation conditions using an electroporator from Biorad trade mark are 2500 volts, 25 μ Farad and 200 Ω . Under these conditions, the mean condenser discharge time is of the order of 4.2 milliseconds. The bacteria are then taken up in 1 ml of SOC medium (ref. CPMB) and shaken for 1 hour at 200 rpm on a rotary shaker in 15 ml Corning tubes. After plating on LB/agar medium supplemented with 100 μ g/ml of carbenicillin, the mini-preparations of the bacteria clones having grown overnight at 37°C are carried out according to the procedure described in "Current Protocols in Molecular Biology". After digestion of the DNA with EcoRI and separation by electrophoresis on a 0.8 % LGTA/TBE agarose gel (ref. CPMB), the clones having a 1.7 kbp insert are conserved. A final verification consists in ensuring that the purified DNA indeed exhibits a hybridization signal with the EPSPS probe from Arabidopsis thaliana. After electrophoresis, the DNA fragments are transferred onto a Hybond N membrane from Amersham according to the Southern procedure described in "Current Protocols in Molecular Biology". The filter is hybridized with the EPSPS probe from Arabidopsis thaliana according to the conditions described in paragraph 3 above. The plasmid clone having a 1.7 kbp insert and hybridizing with the EPSPS probe from Arabidopsis thaliana was prepared on a larger scale and the DNA resulting from the lysis of the bacteria purified on a CsCl gradient as described in "Current

Prot cols in Molecular Biology". The purified DNA was partially sequenced with a Pharmacia kit, following the supplier's instructions and using, as primers, the direct and reverse M13 universal primers ordered from the same supplier. The partial sequence produced covers about 0.5 kbp. The derived amino acid sequence in the region of the mature protein (about 50 amino acid residues) exhibits 100 % identity with the corresponding amino sequence of the mature maize EPSPS described in American patent USP 4,971,908. This clone, corresponding to a 1.7 kbp EcoRI fragment of the DNA for the EPSP from the BMS maize cell suspension, was called pRPA-ML-711. The complete sequence of this clone was obtained on both strands by using the Pharmacia kit procedure and by synthesizing oligonucleotides which are complementary and of opposite direction every 250 bp approximately. The complete sequence of this 1713 bp clone obtained is presented by SEQ ID No. 1.

6. Production of the clone pRPA-ML-715:

Analysis of the sequence of the clone pRPA-ML-711 and in particular comparison of the derived amino acid sequence with that from maize shows a sequence extension of 92 bp upstream of the GCG codon encoding the NH₂-terminal alanine of the mature part of the maize EPSPS (American patent USP 4,971,908). Likewise, a 288 bp extension downstream of the AAT codon encoding the COOH-terminal asparagine of the mature part of the maize EPSPS (American patent USP

4,971,908) is observed. These two parts might correspond, for the NH₂-terminal extension, to a portion of the sequence of a signal peptide before plastid location and, for the COOH-terminal extension, to the untranslated 3' region of the cDNA.

5 untranslatable to 1994
In order to obtain a cDNA encoding the mature
part of the cDNA for the maize EPSPS, as described in
USP 4,971,908, the following operations were carried
out:

10 a) Elimination of the untranslated 3' region:
construction of pRPA-ML-712:

The clone pRPA-ML-711 was cut with the restriction enzyme AseI and the resulting ends of this cut made blunt by treating with the Klenow fragment of DNA polymerase I according to the procedure described in CPMB. A cut with the restriction enzyme SacII was then performed. The DNA resulting from these operations was separated by electrophoresis on a 1% LGTA/TBE agarose gel (ref. CPMB).

20 The gel fragment containing the insert "Asel-
blunt ends/SacII" of 0.4 kbp was excised from the gel
and purified according to the procedure described in
paragraph 5 above. The DNA of the clone pRPA-ML-711 was
25 cut with the restriction enzyme HindIII situated in the
polylinker of the cloning vector pUC19 and the ends
resulting from this cut were made blunt by treating
with the Klenow fragment of DNA polymerase I. A cut
with the restriction enzyme SacII was then performed.

The DNA resulting from these manipulations was separated by electrophoresis on a 0.7 % LGTA/TBE agarose gel (ref. CPMB).

The gel fragment containing the insert 5 HindIII-blunt ends/SacII of about 3.7 kbp was excised from the gel and purified according to the procedure described in paragraph 5 above.

The two inserts were ligated, and 2 μ l of the ligation mixture served to transform E.coli DH10B as 10 described above in paragraph 5.

The plasmid DNA content of the various clones was analysed according to the procedure described for pRPA-ML-711. One of the plasmid clones retained 15 contains an EcoRI-HindIII insert of about 1.45 kbp. The sequence of the terminal ends of this clone shows that the 5' end of the insert corresponds exactly to the corresponding end of pRPA-ML-711 and that the 3' terminal end has the following sequence:

"5'...ATTTAAGCTCTAGAGTCGACCTGCAGGCATGCAAGCTT-3'".

20 The sequence underlined corresponds to the codon for the COOH-terminal amino acid asparagine, the next codon corresponding to the stop codon for translation. The nucleotides downstream correspond to sequence components of the polylinker of pUC19. This 25 clone, comprising the sequence of pRPA-ML-711 up to the site for termination of translation of the mature maize EPSPS and followed by sequences of the polylinker of pUC19 up to the HindIII site, was called pRPA-ML-712.

TOP SECRET EYES ONLY

b) Modification of the 5' end of pRPA-ML-712:
construction of pRPA-ML-715

The clone pRPA-ML-712 was cut with the restriction enzymes PstI and HindIII. The DNA resulting from these manipulations was separated by electrophoresis on a 0.8 % LGTA/TBE agarose gel (ref. CPMB). The gel fragment containing the PstI/EcoRI insert of 1.3 kbp was excised from the gel and purified according to the procedure described in paragraph 5 above. This insert was ligated in the presence of an equimolar quantity of each of the two partially complementary oligonucleotides of sequence:

Oligo 1: 5'-GAGCCGAGCTCCATGGCCGGCGCCGAGGAGATCGTGCTGCA-3'

Oligo 2: 5'-GCACGGATCTCCCTGGCGCCGGCCATGGAGCTGGCTC-3'

as well as in the presence of DNA from the plasmid pUC19 digested with the restriction enzymes BamHI and HindIII.

Two μ l of the ligation mixture served to transform E.coli DH10B as described above in paragraph 5. After analysis of the plasmid DNA content of various clones according to the procedure described above in paragraph 5, one of the clones having an insert of about 1.3 kbp was conserved for subsequent analyses. The sequence of the terminal 5' end of the clone retained shows that the DNA sequence in this region is the following: sequence of the polylinker of pUC19 of the EcoRI to BamHI sites, followed by the sequence of the oligonucleotides used during the cloning, followed

by the rest of the sequence present in pRPAML-712. This clone was called pRPAL-713. This clone has methionine codon ATG included in an NcoI site upstream of the N-terminal alanine codon of the mature EPSPS synthase. Furthermore, the alanine and glycine codons of the N-terminal end were conserved, but modified on the third variable base: initial GCGGAT gives modified GCGGGC.

The clone pRPAL-713 was cut with the restriction enzyme HindIII and the ends of this cut made blunt by treating with the Klenow fragment of DNA polymerase I. A cut with the restriction enzyme SacI was then performed. The DNA resulting from these manipulations was separated by electrophoresis on a 10 0.8 % LGTA/TBE agarose gel (ref. CPMB). The gel fragment containing the insert "HindIII-blunt ends/SacI" of 1.3 kbp was excised from the gel and purified according to the procedure described in paragraph 5 above. This insert was ligated in the 15 presence of DNA from the plasmid pUC19 digested with the restriction enzyme XbaI and the ends of this cut made blunt by treating with the Klenow fragment of DNA polymerase I. A cut with the restriction enzyme SacI was then performed. Two μ l of the ligation mixture 20 served to transform *E.coli* DH10B as described above in paragraph 5. After analysis of the plasmid DNA content of various clones according to the procedure described 25 above in paragraph 5, one of the clones having an

TOP SECRET

insert of about 1.3 kbp was conserved for subsequent analyses. The sequence of the terminal ends of the clone retained shows that the DNA sequence is the following: sequence of the polylinker of pUC19 of the 5 EcoRI to SacI sites, followed by the sequence of the oligonucleotides used during the cloning, from which the 4 bp GATCC of oligonucleotide 1 described above have been deleted, followed by the rest of the sequence present in pRPA-ML-712 up to the HindIII site and 10 sequence of the polylinker of pUC19 from XbaI to HindIII. This clone was called pRPA-ML-715.

7) Production of a cDNA encoding a mature maize EPSPS

All the mutagenesis steps were carried out 15 with the U.S.E. mutagenesis kit from Pharmacia, following the instructions of the supplier. The principle of this mutagenesis system is as follows: the plasmid DNA is heat-denatured and recombined in the presence of a molar excess, on the one hand, of the 20 mutagenesis oligonucleotide and, on the other hand, of an oligonucleotide which makes it possible to eliminate a unique restriction enzyme site present in the polylinker. After the reassociation step, the synthesis of the complementary strand is performed by the action 25 of T4 DNA polymerase in the presence of T4 DNA ligase and protein of gene 32 in an appropriate buffer provided. The synthesis product is incubated in the presence of the restriction enzyme, whose site is

suppos d t have disapp ar d by mutagenesis. The E.coli strain exhibiting, in particular, the mutS mutation is used as host for the transformation of this DNA. After growth in liquid medium, the total plasmid DNA is prepared and incubated in the presence of the restriction enzyme used above. After these treatments, the E.coli DH10B strain is used as host for the transformation. The plasmid DNA of the isolated clones is prepared and the presence of the mutation introduced is checked by sequencing.

10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960 965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560 1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730 1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790 1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845 1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900 1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015 2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415 2420 2425 2430 2435 2440 2445 2450 2455 2460 2465 2470 2475 2480 2485 2490 2495 2500 2505 2510 2515 2520 2525 2530 2535 2540 2545 2550 2555 2560 2565 2570 2575 2580 2585 2590 2595 2600 2605 2610 2615 2620 2625 2630 2635 2640 2645 2650 2655 2660 2665 2670 2675 2680 2685 2690 2695 2700 2705 2710 2715 2720 2725 2730 2735 2740 2745 2750 2755 2760 2765 2770 2775 2780 2785 2790 2795 2800 2805 2810 2815 2820 2825 2830 2835 2840 2845 2850 2855 2860 2865 2870 2875 2880 2885 2890 2895 2900 2905 2910 2915 2920 2925 2930 2935 2940 2945 2950 2955 2960 2965 2970 2975 2980 2985 2990 2995 3000 3005 3010 3015 3020 3025 3030 3035 3040 3045 3050 3055 3060 3065 3070 3075 3080 3085 3090 3095 3100 3105 3110 3115 3120 3125 3130 3135 3140 3145 3150 3155 3160 3165 3170 3175 3180 3185 3190 3195 3200 3205 3210 3215 3220 3225 3230 3235 3240 3245 3250 3255 3260 3265 3270 3275 3280 3285 3290 3295 3300 3305 3310 3315 3320 3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395 3400 3405 3410 3415 3420 3425 3430 3435 3440 3445 3450 3455 3460 3465 3470 3475 3480 3485 3490 3495 3500 3505 3510 3515 3520 3525 3530 3535 3540 3545 3550 3555 3560 3565 3570 3575 3580 3585 3590 3595 3600 3605 3610 3615 3620 3625 3630 3635 3640 3645 3650 3655 3660 3665 3670 3675 3680 3685 3690 3695 3700 3705 3710 3715 3720 3725 3730 3735 3740 3745 3750 3755 3760 3765 3770 3775 3780 3785 3790 3795 3800 3805 3810 3815 3820 3825 3830 3835 3840 3845 3850 3855 3860 3865 3870 3875 3880 3885 3890 3895 3900 3905 3910 3915 3920 3925 3930 3935 3940 3945 3950 3955 3960 3965 3970 3975 3980 3985 3990 3995 4000 4005 4010 4015 4020 4025 4030 4035 4040 4045 4050 4055 4060 4065 4070 4075 4080 4085 4090 4095 4100 4105 4110 4115 4120 4125 4130 4135 4140 4145 4150 4155 4160 4165 4170 4175 4180 4185 4190 4195 4200 4205 4210 4215 4220 4225 4230 4235 4240 4245 4250 4255 4260 4265 4270 4275 4280 4285 4290 4295 4300 4305 4310 4315 4320 4325 4330 4335 4340 4345 4350 4355 4360 4365 4370 4375 4380 4385 4390 4395 4400 4405 4410 4415 4420 4425 4430 4435 4440 4445 4450 4455 4460 4465 4470 4475 4480 4485 4490 4495 4500 4505 4510 4515 4520 4525 4530 4535 4540 4545 4550 4555 4560 4565 4570 4575 4580 4585 4590 4595 4600 4605 4610 4615 4620 4625 4630 4635 4640 4645 4650 4655 4660 4665 4670 4675 4680 4685 4690 4695 4700 4705 4710 4715 4720 4725 4730 4735 4740 4745 4750 4755 4760 4765 4770 4775 4780 4785 4790 4795 4800 4805 4810 4815 4820 4825 4830 4835 4840 4845 4850 4855 4860 4865 4870 4875 4880 4885 4890 4895 4900 4905 4910 4915 4920 4925 4930 4935 4940 4945 4950 4955 4960 4965 4970 4975 4980 4985 4990 4995 5000 5005 5010 5015 5020 5025 5030 5035 5040 5045 5050 5055 5060 5065 5070 5075 5080 5085 5090 5095 5100 5105 5110 5115 5120 5125 5130 5135 5140 5145 5150 5155 5160 5165 5170 5175 5180 5185 5190 5195 5200 5205 5210 5215 5220 5225 5230 5235 5240 5245 5250 5255 5260 5265 5270 5275 5280 5285 5290 5295 5300 5305 5310 5315 5320 5325 5330 5335 5340 5345 5350 5355 5360 5365 5370 5375 5380 5385 5390 5395 5400 5405 5410 5415 5420 5425 5430 5435 5440 5445 5450 5455 5460 5465 5470 5475 5480 5485 5490 5495 5500 5505 5510 5515 5520 5525 5530 5535 5540 5545 5550 5555 5560 5565 5570 5575 5580 5585 5590 5595 5600 5605 5610 5615 5620 5625 5630 5635 5640 5645 5650 5655 5660 5665 5670 5675 5680 5685 5690 5695 5700 5705 5710 5715 5720 5725 5730 5735 5740 5745 5750 5755 5760 5765 5770 5775 5780 5785 5790 5795 5800 5805 5810 5815 5820 5825 5830 5835 5840 5845 5850 5855 5860 5865 5870 5875 5880 5885 5890 5895 5900 5905 5910 5915 5920 5925 5930 5935 5940 5945 5950 5955 5960 5965 5970 5975 5980 5985 5990 5995 6000 6005 6010 6015 6020 6025 6030 6035 6040 6045 6050 6055 6060 6065 6070 6075 6080 6085 6090 6095 6100 6105 6110 6115 6120 6125 6130 6135 6140 6145 6150 6155 6160 6165 6170 6175 6180 6185 6190 6195 6200 6205 6210 6215 6220 6225 6230 6235 6240 6245 6250 6255 6260 6265 6270 6275 6280 6285 6290 6295 6300 6305 6310 6315 6320 6325 6330 6335 6340 6345 6350 6355 6360 6365 6370 6375 6380 6385 6390 6395 6400 6405 6410 6415 6420 6425 6430 6435 6440 6445 6450 6455 6460 6465 6470 6475 6480 6485 6490 6495 6500 6505 6510 6515 6520 6525 6530 6535 6540 6545 6550 6555 6560 6565 6570 6575 6580 6585 6590 6595 6600 6605 6610 6615 6620 6625 6630 6635 6640 6645 6650 6655 6660 6665 6670 6675 6680 6685 6690 6695 6700 6705 6710 6715 6720 6725 6730 6735 6740 6745 6750 6755 6760 6765 6770 6775 6780 6785 6790 6795 6800 6805 6810 6815 6820 6825 6830 6835 6840 6845 6850 6855 6860 6865 6870 6875 6880 6885 6890 6895 6900 6905 6910 6915 6920 6925 6930 6935 6940 6945 6950 6955 6960 6965 6970 6975 6980 6985 6990 6995 7000 7005 7010 7015 7020 7025 7030 7035 7040 7045 7050 7055 7060 7065 7070 7075 7080 7085 7090 7095 7100 7105 7110 7115 7120 7125 7130 7135 7140 7145 7150 7155 7160 7165 7170 7175 7180 7185 7190 7195 7200 7205 7210 7215 7220 7225 7230 7235 7240 7245 7250 7255 7260 7265 7270 7275 7280 7285 7290 7295 7300 7305 7310 7315 7320 7325 7330 7335 7340 7345 7350 7355 7360 7365 7370 7375 7380 7385 7390 7395 7400 7405 7410 7415 7420 7425 7430 7435 7440 7445 7450 7455 7460 7465 7470 7475 7480 7485 7490 7495 7500 7505 7510 7515 7520 7525 7530 7535 7540 7545 7550 7555 7560 7565 7570 7575 7580 7585 7590 7595 7600 7605 7610 7615 7620 7625 7630 7635 7640 7645 7650 7655 7660 7665 7670 7675 7680 7685 7690 7695 7700 7705 7710 7715 7720 7725 7730 7735 7740 7745 7750 7755 7760 7765 7770 7775 7780 7785 7790 7795 7800 7805 7810 7815 7820 7825 7830 7835 7840 7845 7850 7855 7860 7865 7870 7875 7880 7885 7890 7895 7900 7905 7910 7915 7920 7925 7930 7935 7940 7945 7950 7955 7960 7965 7970 7975 7980 7985 7990 7995 8000 8005 8010 8015 8020 8025 8030 8035 8040 8045 8050 8055 8060 8065 8070 8075 8080 8085 8090 8095 8100 8105 8110 8115 8120 8125 8130 8135 8140 8145 8150 8155 8160 8165 8170 8175 8180 8185 8190 8195 8200 8205 8210 8215 8220 8225 8230 8235 8240 8245 8250 8255 8260 8265 8270 8275 8280 8285 8290 8295 8300 8305 8310 8315 8320 8325 8330 8335 8340 8345 8350 8355 8360 8365 8370 8375 8380 8385 8390 8395 8400 8405 8410 8415 8420 8425 8430 8435 8440 8445 8450 8455 8460 8465 8470 8475 8480 8485 8490 8495 8500 8505 8510 8515 8520 8525 8530 8535 8540 8545 8550 8555 8560 8565 8570 8575 8580 8585 8590 8595 8600 8605 8610 8615 8620 8625 8630 8635 8640 8645 8650 8655 8660 8665 8670 8675 8680 8685 8690 8695 8700 8705 8710 8715 8720 8725 8730 8735 8740 8745 8750 8755 8760 8765 8770 8775 8780 8785 8790 8795 8800 8805 8810 8815 8820 8825 8830 8835 8840 8845 8850 8855 8860 8865 8870 8875 8880 8885 8890 8895 8900 8905 8910 8915 8920 8925 8930 8935 8940 8945 8950 8955 8960 8965 8970 8975 8980 8985 8990 8995 9000 9005 9010 9015 9020 9025 9030 9035 9040 9045 9050 9055 9060 9065 9070 9075 9080 9085 9090 9095 9100 9105 9110 9115 9120 9125 9130 9135 9140 9145 9150 9155 9160 9165 9170 9175 9180 9185 9190 9195 9200 9205 9210 9215 9220 9225 9230 9235 9240 9245 9250 9255 9260 9265 9270 9275 9280 9285 9290 9295 9300 9305 9310 9315 9320 9325 9330 9335 9340 9345 9350 9355 9360 9365 9370 9375 9380 9385 9390 9395 9400 9405 9410 9415 9420 9425 9430 9435 9440 9445 9450 9455 9460 9465 9470 9475 9480 9485 9490 9495 9500 9505 9510 9515 9520 9525 9530 9535 9540 9545 9550 9555 9560 9565 9570 9575 9580 9585 9590 9595 9600 9605 9610 9615 9620 9625 9630 9635 9640 9645 9650 9655 9660 9665 9670 9675 9680 9685 9690 9695 9700 9705 9710 9715 9720 9725 9730 9735 9740 9745 9750 9755 9760 9765 9770 9775 9780 9785 9790 9795 9800 9805 9810 9815 9820 9825 9830 9835 9840 9845 9850 9855 9860 9865 9870 9875 9880 9885 9890 9895 9900 9905 9910 9915 9920 9925 9930 9935 9940 9945 9950 9955 9960 9965 9970 9975 9980 9985 9990 9995 9999 10000 10005 10010 10015 10020 10025 10030 10035 10040 10045 10050 10055 10060 10065 10070 10075 10080 10085 10090 10095 10100 10105 10110 10115 10120 10125 10130 10135 10140 10145 10150 10155 10160 10165 10170 10175 10180 10185 10190 10195 10200 10205 10210 10215 10220 10225 10230 10235 10240 10245 10250 10255 10260 10265 10270 10275 10280 10285 10290 10295 10300 10305 10310 10315 10320 10325 10330 10335 10340 10345 10350 10355 10360 10365 10370 10375 10380 10385 10390 10395 10400 10405 10410 10415 10420 10425 10430 10435 10440 10445 10450 10455 10460 10465 10470 10475 10480 10485 10490 10495 10500 10505 10510 10515 10520 10525 10530 10535 10540 10545 10550 10555 10560 10565 10570 10575 10580 10585 10590 10595 10600 10605 10610 10615 10620 10625 10630 10635 10640 10645 10650 10655 10660 10665 10670 10675 10680 10685 10690 10695 10700 10705 10710 10715 10720 10725 10730 10735 10740 10745 10750 10755 10760 10765 10770 10775 10780 10785 10790 10795 10800 10805 10810 10815 10820 10825 10830 10835 10840 10845 10850 10855 10860 10865 10870 10875 10880 10885 10890 10895 10900 10905 10910 10915 10920 10925 10930 10935 10940 10945 10950 10955 10960 10965 10970 10975 10980 10985 10990 10995 11000 11005 11010 11015 11020 11025 11030 11035 11040 11045 11050 11055 11060 11065 11070 11075 11080 11085 11090 11095 11100 11105 11110 11115 11120 11125 11130 11135 11140 11145 11150 11155 1

The 1340 bp sequence of this clone is represented as SEQ ID No. 2 and SEQ ID No. 3.

5 B) Sequence modifications allowing an increase in the resistance character of maize EPSPS to products which are competitive inhibitors of the activity of EPSP synthase.

The following oligonucleotides were used:

a) Thr 102 \rightarrow Ile mutation.

5'-GAATGCTGGAATCGCAATGCGGCCATTGACAGC-3'

10 b) Pro 106 \rightarrow Ser mutation.

5'-GAATGCTGGAACTGCAATGCGGTCTTGACAGC-3'

c) Gly 101 \rightarrow Ala and Thr 102 \rightarrow Ile mutations.

5'-CTTGGGAAATGCTGCCATCGCAATGCGGCCATTG-3'

d) Thr 102 \rightarrow Ile and Pro 106 \rightarrow Ser mutations.

15 5'-GGGGAAATGCTGGAATCGCAATGCGGTCTTGACAGC-3'

After sequencing, the sequence read after

20 mutagenesis on the three mutated fragments is identical to the sequence of the parental DNA pRPA-ML-716 with the exception of the mutagenesis region which corresponds to that of the mutagenesis oligonucleotides used. These clones were called: pRPA-ML-717 for the Thr 102 \rightarrow Ile mutation, pRPA-ML-718 for the Pro 106 \rightarrow Ser mutation, pRPA-ML-719 for the Gly 101 \rightarrow Ala and Thr 102

→ Ile mutations and pRPA-ML-720 for the Thr 102 → Ile and Pro 106 → Ser mutations.

The 1340 bp sequence of pRPA-ML-720 is represented as SEQ ID No. 4 and SEQ ID No. 5.

5 The NcoI-HindIII insert of 1395 bp will be
called in the rest of the descriptions "the double
mutant of maize EPSPS".

EXAMPLE 2: Construction of chimeric genes

The construction of chimeric genes according

10 to the invention is carried out using the following
elements:

1). The genomic clone (cosmid clone c22) from *Arabidopsis thaliana*, containing two genes of the "H3.3-like" type was isolated as described in Chaubet et al. (J. Mol. Biol. 1992. 225 569-574).

2). Intron No. 1:

A DNA fragment of 418 base pairs is purified from digestion of the cosmid clone c22 with the

20 Klenow fragment of DNA polymerase from E.coli, according to the manufacturer's instructions for creating a blunt-ended DNA fragment and then cut with MseI. The purified DNA fragment is ligated to a synthetic oligonucleotide adaptor having the following

25 sequence:

The ligation product is cloned into
 pGEM7zf(+) (Stratagene catalogue No. P2251) which was
 digested with SmaI. This clone, called "intron No. 1",
 is checked by sequencing (SEQ ID No. 6).

5 3). Intron No. 2:

A DNA fragment of 494 base pairs is purified
 from the digestion of the cosmid clone c22 with the
 restriction enzymes AluI and CfoI. The purified DNA
 fragment is ligated to a synthetic oligonucleotide
 10 adaptor having the following sequence:

Adaptor 2: 5' CAGATCCCGGGATCTGCG 3'
 GCGTCTAGGGCCCTAGACGC

The ligation product is cloned into
 pGEM7zf(+) (Stratagene catalogue No. P2251) which was
 15 digested with SmaI. This clone, called "intron No. 2",
 is checked by sequencing (SEQ ID No. 7).

4). pRA-1

The construction of this plasmid is described
 in French patent 9,308,029. This plasmid is a
 20 derivative of pBI 101.1 (Clonetech catalogue No. 6017-
 1) which contains the histone promoter from Arabidopsis
 H4A748 regulating the synthesis of the E.coli β -
 glucuronidase gene and of the nopaline synthase ("NOS")
 polyadenylation site. Thus, a chimeric gene is obtained
 25 having the structure:

"H4A748 promoter-GUS gene-NOS"

5). pCG-1

F0023365 - 1 - 2 - 102

This plasmid contains the above intron No. 1 placed between the H4A74S promoter and the GUS coding region of pRA-1. This plasmid is obtained by digestion of cosmid clone c22 with BamHI and SmaI. The intron No. 1 of 418 base pairs is directly ligated into pRA-1 which was digested with BamHI and SmaI.

Thus, a chimeric gene is obtained having the structure:

"H4A748 promoter-intron No. 1-GUS gene-NOS"

10 6) . pcG-13

This plasmid contains the above intron No. 2 placed between the H4A748 promoter and the GUS coding region of pRA-1. This plasmid is obtained by digestion of cosmid clone c22 with BamHI and SmaI. The intron No. 2 of 494 base pairs is directly ligated into pRA-1 which was digested with BamHI and SmaI.

Thus, a chimeric gene is obtained having the structure:

"H4A748 promoter-intron No. 2-GUS gene-NOS"

20 7). PCG-15

This plasmid contains only intron No. 1 before the above GUS coding sequence placed between the H4A748 promoter and the GUS coding region of pCG-1. This plasmid is obtained by digestion of pCG-1 with 25 BamHI and HindIII followed by treatment with a Klenow fragment of DNA polymerase from E.coli, according to the manufacturer's instructions for creating a blunt-ended DNA fragment.

This vector is then re-ligated to give a chimeric gene having the structure:

"Intron No. 1-GUS-NOS"

8). pcg-18

5 This plasmid contains only the above intron
No. 2 in front of the GUS coding sequence of pCG-13.
This plasmid is obtained by partial digestion of pCG-13
with BamHI and SphI, followed by treatment with a
fragment of T4 phage DNA polymerase, according to the
10 manufacturer's instructions in order to create a blunt-
ended DNA fragment.

This vector is then religated and checked by enzymatic digestion in order to give a chimeric gene having the structure:

15 "intren No. 2-GUS-NOS"

9) - PRPA-RD-124

Addition of a "nos" polyadenylation signal to
pRPA-ML-720 with creation of a cloning cassette
containing the maize double mutant EPSPS gene (Thr 102
20 → Ile and Pro 106 → Ser). pRPA-ML-720 is digested with
HindIII and treated with the Klenow fragment of DNA
polymerase from *E.coli* in order to produce a blunt end.
A second digestion is carried out with NcoI and the
25 EPSPS fragment is purified. The EPSPS gene is then
ligated with purified pRPA-RD-12 (a cloning cassette
containing the nopaline synthase polyadenylation
signal) to give pRPA-RD-124. To obtain the purified
useful vector pRPA-RD-12, it was necessary for the

latter to be previously digested with *Sall*, treated with Klenow DNA polymerase, and then digested a second time with *NcoI*.

10). pRPA-RD-125

5 Addition of an optimized signal peptide (OSP) from pRPA-RD-124 with creation of a cloning cassette containing the EPSPS gene targeted on the plasmids. pRPA-RD-7 (European Patent Application EP 652 286) is digested with *SphI*, treated with T4 DNA polymerase and 10 then digested with *SpeI* and the OSP fragment is purified. This OSP fragment is cloned into pRPA-RD-124 which was previously digested with *NcoI*, treated with Klenow DNA polymerase in order to remove the 3' protruding part, and then digested with *SpeI*. This 15 clone is then sequenced in order to ensure the correct translational fusion between the OSP and the EPSPS gene. pRPA-RD-125 is then obtained.

11). pRPA-RD-196

In this plasmid, the "intron No. 1 + β -glucuronidase gene from *E.coli*" portion of PCG-1 is replaced by a chimeric gene of 2 kilobases containing an optimized signal peptide, a double mutant EPSPS gene (Ile₁₀₂+Ser₁₀₆) and a nopaline synthase polyadenylation site ("NOS") isolated from pRPA-RD-125. To obtain pRPA-25 RD-196, the digestion of PCG-1 is performed with *EcoRI* and *BamHI*, followed by treatment with a Klenow fragment of DNA polymerase from *E.coli*, according to the manufacturer's instructions in order to create a blunt-

and a DNA fragm nt. Th 2-kilobase DNA fragment containing an optimized signal peptide of a d ubl mutant EPSPS gene (Ile₁₀₂+Ser₁₀₆) and a nopaline synthase polyadenylation site ("NOS") is obtained from pRPA-RD-125 by digestion with NcoI and NotI, followed by treatment with DNA polymerase from E.coli, according to the manufacturer's instructions in order to create a blunt-ended DNA fragment. This blunt-ended fragment is then ligated into pCG-1 prepared above.

10 A chimeric gene is thus obtained having the structure:

"H4A748 promoter-OSP-maize EPSPS gene-NOS"

12). pRPA-RD-197

In this plasmid, the " β -glucuronidase gene

15 from E.coli" portion of pCG-1 is replaced by a chimeric gene of 2 kilobases containing an optimized signal peptide, a double mutant EPSPS gene (Ile₁₀₂+Ser₁₀₆) and a nopaline synthase polyadenylation site ("NOS") isolated from pRPA-RD-125. To obtain pRPA-RD-197, the digestion 20 of pCG-1 is performed with EcoRI, followed by treatment with a Klenow fragment of DNA polymerase from E.coli, according to the manufacturer's instructions in order to create a blunt-ended DNA fragment, then cut with SmaI. The 2-kilobase DNA fragment containing an 25 optimized signal peptide, a double mutant EPSPS gene (Ile₁₀₂+Ser₁₀₆) and a nopaline synthase polyadenylation site ("NOS") is obtained from pRPA-RD-125 by digestion with NcoI and NotI, followed by a treatment with DNA

polymerase from E.coli, acc rding to the manufacturer's instructions in order to create a blunt-ended DNA fragment. This blunt-ended fragment is then ligated into pcG-1 prepared above.

5 A chimeric gene is thus obtained having the
structure:

"H4A748 promoter-intron No. 1-maize EPSPS

gene-nos"

"H4A748 promoter mutation .0.1 M 1000

13). PRPA-RD-198

10 In this plasmid, the " β -glucuronidase gene
from E.coli" portion of pCG-13 is replaced by a
chimeric gene of 2 kilobases containing an optimized
signal peptide, a double mutant EPSPS gene (Ile₁₀₂+Ser₁₀₆)
and a nopaline synthase polyadenylation site ("NOS")
15 isolated from pRPA-RD-125. To obtain pRPA-RD-198, the
digestion of pCG-13 is performed with EcoRI, followed
by treatment with a Klenow fragment of DNA polymerase
from E.coli, according to the manufacturer's
instructions in order to create a blunt-ended DNA
fragment, then cut with SmaI. The 2-kilobase DNA
20 fragment containing an optimized signal peptide, a
double mutant EPSPS gene (Ile₁₀₂+Ser₁₀₆) and a nopaline
synthase polyadenylation site ("NOS") is obtained from
pRPA-RD-125 by digestion with NcoI and NotI, followed
25 by a treatment with DNA polymerase from E.coli,
according to the manufacturer's instructions in order

t create a blunt-ended DNA fragment. This blunt-ended fragment is then ligated into pCG-13 prepared above.

A chimeric gene is thus obtained having the structure:

5 "H4A748 promoter-intron No. 2-OSP-maize EPSPS gene-NOS"

EXAMPLE 3: Expression of the activity of a reporter gene

1) Transformation and regeneration

The vector is introduced into the

10 nonencogenic strain of Agrobacterium tumefaciens LBA 4404 available from a catalogue (Clontech #6027-1) by triparental crossing using the "helper" plasmid pRK 2013 in Escherichia coli HB101 according to the procedure described by Bevan M. (1984) Nucl. Acids Res., 12, 8711-8721.

The transformation technique using root explants of Arabidopsis thaliana L.-ecotype C24 was carried out according to the procedure described by Valvekens D. et al. (1988) Proc. Natl. Acad. Sci USA, 85, 5536-5540. Briefly, 3 steps are necessary:

induction of the formation of calli on Gamborg B5 medium supplemented with 2,4-D and kinetin; formation of buds on Gamborg B5 medium supplemented with 2iP and IAA; rooting and formation of seeds on hormone-free MS.

25 2) Measurement of the GUS activity in plants

a - histochemical observations

visualization of the GUS activity by histochemical spots (Jefferson R.A. et al. (1987) EMBO

J., 6, 3901-3907) on 10-day transgenic plants shows an increase in the intensity of the hist. chemical pattern which is tissue-specific for the plasmids containing the intron sequences (pCG-1 and pCG-13) compared with those without these introns (pRA-1). In particular, the pattern of spots for pCG-1 and pCG-13 is identical, showing an increase in intensity of the spots for the vascular and meristematic tissues, leaves and roots compared with that of the construct pRA-1. The constructs containing only the sequences of intron No. 1 (pCG-15 and pCG-18) show an extremely clear histochemical spot only in the apical meristem region.

b - fluorometric measurements

The GUS activity measured by fluorometry on extracts of floral and leaf buds of the rosette (Jefferson R.A. et al. (1987) EMBO J., 6, 3901-3907) from 12 plants, shows that the activity of the H4A748 promoter is increased under the influence of intron Nos. 1 and 2. Compared with the construct pRA-1, the GUS activity of pCG-1 and pCG-13 are at least six times greater in the floral buds, twenty times greater in the leaves of the rosette and twenty-six times greater in the roots.

These measurements clearly show that introns Nos. 1 and 2 of *Arabidopsis* histone genes of the "H3.3-like" type used as a regulatory element induces an increase in the activity of expression of the chimeric gene.

EXAMPLE 4: Tolerance of transgenic plants to a herbicide

1) Transformation and regeneration

The vector is introduced into the

5 nononcogenic strain of Agrobacterium tumefaciens LBA
4404 available from a catalogue (Clontech #6027-1) by
triparental crossing using the "helper" plasmid pRK
2013 in Escherichia coli HB101 according to the
procedure described by Bevan M. (1984) Nucl. Acids
Res., 12, 8711-8721.

The transformation technique using foliar explants of tobacco is based on the procedure described by Horsh R. et al. (1985) *Science*, 227, 1229-1231. The regeneration of the PBD6 tobacco (origin SEITA-France) from foliar explants is carried out on a Murashige and Skoog (MS) basal medium comprising 30 g/l of sucrose as well as 200 µg/ml of kanamycin in three successive steps: the first comprises the induction of shoots on an MS medium supplemented with 30 g of sucrose containing 0.05 mg of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 15 days. The shoots formed during this step are then developed by culturing on an MS medium supplemented with 30 g/l of sucrose but not containing any hormone, for 10 days. The developed shoots are then removed and they are cultured on an MS rooting medium diluted one half, with half the content of salts, vitamins and sugars and not containing any

THE DILEMMA IN THE ECONOMY

harm n . After about 15 days, the ro t d sh ts ar planted in the soil.

2) Measurement of the tolerance to glyphosate:

5 Twenty transformed plants were regenerated and transferred to a greenhouse for each of the constructs pRPA-RD-196, pRPA-RD-197 and pRPA-RD-198. These plants were treated in a greenhouse at the 5-leaf stage with an aqueous suspension of herbicide, sold 10 under the trademark RoundUp, corresponding to 0.8 kg of active substance glyphosate per hectare.

The results correspond to the observation of phytotoxicity values noted 3 weeks after treatment. Under these conditions, it is observed that the plants 15 transformed with the constructs have on average an acceptable tolerance (pRPA-RD-196) or even a good tolerance (pRPA-RD-197 and pRPA-RD-198) whereas the untransformed control plants are completely destroyed.

These results show clearly the improvement 20 offered by the use of a chimeric gene according to the invention for the same gene encoding tolerance to glyphosate.

The transformed plants according to the invention may be used as parents for producing lines 25 and hybrids having the phenotypic character corresponding to the expression of the chimeric gene introduced.

FOURTY-SEVEN - FIVE EIGHT EIGHT